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Eighth day of June 2011

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P/00/009
Regulation 3.2

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: "SKIN REGENERATION SYSTEM"

The invention is described in the following statement:

TITLE

SKIN REGENERATION SYSTEM

FIELD OF THE INVENTION

THIS INVENTION relates to skin regeneration and, more particularly, to a medium, system and method for propagating keratinocytes for subsequent use in skin growth and regeneration. This invention also relates to compositions for use in skin growth and regeneration *in situ*.

BACKGROUND OF THE INVENTION

The insulin-like growth factors (IGFs), IGF-I and IGF-II, are mitogenic peptide growth factors involved in a broad range of cellular processes including hyperplasia, DNA synthesis, differentiation, cell cycle progression and inhibition of apoptosis (Keiss *et al.*, 1994, Hormone Research **41** 66; Wood & Yee, 2000, J. Mammary Gland Biology and Neoplasia **5** 1; Jones & Clemmons, 1995, Endocrine Rev. **16** 3). These effects are mediated through binding to their tyrosine-kinase linked cell surface receptor, the type 1 IGF receptor (IGF-IR). The IGFs are also tightly regulated by a family of specific binding proteins, termed IGFBPs, whose primary role is to bind free IGFs and thereby moderate their half-life, specificity and activity (Clemmons, 1998, Mol. Cell. Endocrinol. **140** 19).

Recently, vitronectin (VN) has been shown to bind directly to IGF-II (Upton et al., 1999. Endocrinology **140** 2928-31) while IGF-I can bind to VN in the presence of certain IGFBPs (International Publication WO 02/24219; Kricker *et al.*, 2003, Endocrinol. **144** 2807-15). The finding that VN, an ECM organization and adhesion molecule, binds IGF-II with an affinity that is similar to that of IGF-II for IGF-IR (Upton *et al.*, 1999, *supra*), its biologically relevant receptor, reveals a specific

physical link between IGF action and VN in the ECM. In addition, IGF-II bound to VN, and IGF-I bound to VN via IGFBPs, can stimulate synergistic functional responses in a diverse range of cells including human keratinocytes *in vitro* (International Publication WO 02/24219; Noble *et al.*, 2003, *supra*; Kricker *et al.*, 2003, *supra*).

Wounds, burns and ulcers are debilitating and painful skin conditions that require intensive and costly treatments which, in many cases, are only partly successful. For example, more than 520,000 Australians are currently diagnosed with diabetes, and of these, more than 5% will experience foot ulcers. These wounds significantly compromise the quality of life of the patient, often lead to prolonged hospitalisation, and may ultimately result in amputation. In fact, the vast majority of lower limb amputations performed are attributed to a non-healing ulcer.

An increasingly preferred approach to healing wounds, burns and ulcers is to replace dead or damaged skin with autologous or allogeneic keratinocytes grown *in vitro*. Typically, keratinocytes are grown in defined media in the presence of exogenous factors such as serum or bovine pituitary extracts, usually with feeder cells that optimize keratinocyte growth.

SUMMARY OF THE INVENTION

Prior art keratinocyte growth systems are relatively expensive by virtue of the inclusion of the aforementioned exogenous factors and feeder cells. Furthermore, animal-derived exogenous factors such as serum, bovine pituitary extracts and feeder cells are relatively poorly defined and may harbour infectious agents such as those that cause CJD, HIV and other diseases. To this end, the present inventors have discovered that protein complexes comprising IGF-II and VN or IGF-I and IGFBP

and VN stimulate significant proliferative responses in primary cultures of keratinocytes *ex vivo* in the absence of serum and cell feeder layers that are typically required for keratinocyte growth *ex vivo*. More particularly, protein complexes comprising IGF-II and VN or IGF-I and IGFBP and VN can be used to enhance keratinocyte growth for the purposes of skin replacement, burn and wound healing and other therapeutic treatments that require skin growth *ex vivo*.

Therefore, in a first aspect, the invention provides a keratinocyte or keratinocyte progenitor cell culture medium comprising:

(i) at least an IGF selected from IGF-I and IGF-II; and

(ii) an absence of serum or an amount of serum which in the absence of said at least an IGF would not support keratinocyte growth.

In a second aspect, the invention provides a keratinocyte or keratinocyte progenitor cell culture system comprising:

(i) a keratinocyte culture medium according to the first aspect;

(ii) a culture vessel; and

(ii) an absence of a feeder cell layer.

In a third aspect, the invention provides a method of culturing keratinocytes or keratinocyte progenitor cells including the step of growing one or more keratinocytes in a culture medium comprising at least an IGF and in the absence of serum or in the presence of an amount of serum which in the absence of said at least an IGF would not support keratinocyte growth.

In a fourth aspect, the invention provides a method of culturing keratinocytes or keratinocyte progenitor cells including the step of growing one or more

keratinocytes in a culture medium comprising at least an IGF and in the absence of feeder cells.

In a fifth aspect, the invention provides a method of culturing keratinocytes or keratinocyte progenitor cells including the step of growing one or more keratinocytes in a culture medium comprising at least an IGF and in the absence of:

(i) serum or in the presence of an amount of serum which in the absence of said at least an IGF would not support keratinocyte growth; and

(ii) feeder cells.

In a sixth aspect, the invention provides a keratinocyte or keratinocyte progenitor cells capable of expressing at least one recombinant protein selected from the group consisting of:

(i) a recombinant IGF;

(ii) a recombinant IGFBP;

(iii) a recombinant vitronectin;

(iv) a recombinant chimeric protein comprising at least a type-1 IGR-binding fragment of IGF-I or IGF-II; and

(v) a recombinant chimeric molecule comprising at least an α-v integrin binding fragment of VN

Suitably, the recombinant proteins set forth in (i) to (iii) expressed by said keratinocyte or keratinocyte progenitor cell are capable of forming an isolated protein complex comprising:

(a) IGF-I, an IGFBP and vitronectin; or

(b) IGF-II and vitronectin.

In a seventh aspect, the invention provides a pharmaceutical composition for aerosol delivery of keratinocytes or keratinocyte progenitor cells comprising one or more keratinocytes and at least an IGF together with a pharmaceutically acceptable carrier, diluent or excipient.

In an eighth aspect, the invention provides a method of delivering keratinocytes or keratinocyte progenitor cells for skin regeneration *in situ* including the step of spraying one or more keratinocytes onto the skin of an individual to facilitate skin regeneration.

In a ninth aspect, the invention provides a method of regenerating skin *in situ*, including the steps of:

(i) spraying one or more keratinocytes or keratinocyte progenitor cells onto the skin of an individual;

and

(ii) growing said keratinocytes or keratinocyte progenitor cells to form regenerated skin *in situ*.

In embodiments where IGF-I is present, it is preferred that IGF-I is a component of a protein complex further comprising an IGFBP and VN.

In embodiments where IGF-II is present, it is preferred that IGF-II is a component of a protein complex further comprising VN in the absence of an IGFBP.

The invention further contemplates use of synthetic proteins comprising at least a type-1 IGR-binding fragment of IGF-I or IGF-II and an α_v integrin-binding domain of VN.

Preferably, the integrin is an $\alpha_v\beta_3$ integrin or an $\alpha_v\beta_5$ integrin.

Throughout this specification, unless otherwise indicated, "comprise", "comprises" and "comprising" are used inclusively rather than exclusively, so that a stated integer or group of integers may include one or more other non-stated integers or groups of integers.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. IGF protein complexes support the *ex vivo* expansion of keratinocytes. Keratinocytes derived from adult human skin seeded onto IGF protein complexes survive and grow at rates comparable to cells seeded onto irradiated mouse 3T3 cells in the presence of foetal bovine serum. Cell growth was observed by: (a) visual examination of culture morphology/confluence; and (b) quantified by MTT assay. (a) from left to right: feeder layer + bovine serum; control without feeder layer or serum; IGF-I + IGFBP5 + VN without feeder layer or serum; (b) left to right: feeder layer + bovine serum; feeder layer alone; IGF-I + IGFBP5 + VN without feeder layer or serum; IGF-I + N129DIGFBP3 mutant + VN. VN is present at 300 ng/well. IGF-I or IGF-II are present at 100 ng/well and IGFBPs are present at 300 ng/well.

Figure 2. IGF protein complexes supplemented with other growth factors further enhance growth of cultures of keratinocytes. Keratinocytes derived from adult human skin were cultured on IGF protein complexes plus epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) and assayed for protein synthesis by [^{3}H]-leucine incorporation. Cells seeded on trimeric IGF-I, IGFBP5 and VN or dimeric IGF-II and VN protein complexes grow at rates equivalent to Defined Keratinocyte Media (DKM, Invitrogen). IGF protein complexes further incorporating EGF (100 ng/well) and bFGF (100 ng/well) significantly enhanced protein synthesis

compared to DKM ($p<0.05$). IGF-I or IGF-II are present at 100 ng/well, VN at 300 ng/well and IGFBPs are present at 300 ng/well.

DETAILED DESCRIPTION OF THE INVENTION

The present invention has arisen from the discovery that isolated protein complexes comprising IGF-II and VN or IGF-I and IGFBP and VN stimulate significant proliferative responses in primary cultures of keratinocytes *ex vivo* in the absence of serum and cell feeder layers that are typically required for keratinocyte growth *ex vivo*.

This invention therefore provides technology that improves current clinical best practice for *ex vivo* skin regeneration. In addition, the present invention also provides for the derivation and establishment of keratinocytes from tissue biopsies. In a preferred form, the invention provides a keratinocyte culture medium and system that utilizes autologous vitronectin isolated from a patient's own serum or produced recombinantly, thereby further minimizing the use of xenogeneic or allogeneic support systems, as well as eliminating use of poorly-defined supplementary products. This will therefore provide an autologous-cell based tissue engineering system that can be translated to approved therapeutic applications.

For the purposes of this invention, by "*isolated*" is meant material that has been removed from its natural state or otherwise been subjected to human manipulation. Isolated material may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state. Isolated material may be in native, chemical synthetic or recombinant form.

As used herein, by "*synthetic*" is meant not naturally occurring but made through human technical intervention. In the context of synthetic proteins and nucleic acids, this encompasses molecules produced by recombinant or chemical synthetic and combinatorial techniques as are well understood in the art.

By "*protein*" is meant an amino acid polymer. The amino acids may be natural or non-natural amino acids, D- or L- amino acids as are well understood in the art.

A "*peptide*" is a protein having less than fifty (50) amino acids.

A "*polypeptide*" is a protein having fifty (50) or more amino acids.

In particular aspects, the invention provides a keratinocyte culture medium and system comprising at least IGF-I and/or IGF-II, such that exogenous, animal-derived factors such as feeder cells and/or serum are not required or are required at substantially reduced levels whereby keratinocyte growth and/or viability are maintained.

Keratinocytes and/or their progenitors may be derived from normal skin, skin biopsies such as obtained from wounds or ulcers or from outer root sheath (ORS) cells of hair follicles, although without limitation thereto.

In particular embodiments, the keratinocyte culture medium and system uses isolated protein complexes comprising at least IGF-I or IGF-II.

In embodiments where IGF-I is present, it is preferred that IGF-I is a component of an protein complex further comprising and IGFBP and vitronectin (VN).

The IGFBP is selected from IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP5 and IGFBP6.

Preferably, the IGFBP is IGFBP3 or IGFBP5.

In embodiments where IGF-II is present, it is preferred that IGF-II is a component of an isolated protein complex further comprising vitronectin (VN).

It will also be appreciated that vitronectin (VN) may be in monomeric or multimeric form.

In a preferred embodiment, the isolated protein complexes comprise autologous, purified VN.

Preferably, keratinocytes are cultured in culture vessels as typically used in the art. It will therefore be appreciated that the respective amounts of IGFs, VN and IGFBPs present during culture will depend on factors such as the size of the culture vessel, amount of liquid medium present in the vessel, cell density and other factors known in the art.

For guidance, in a 1.9 cm^2 well, preferred amounts are as follows:

VN: 50-5000 ng or advantageously 100-500 ng;

IGF: 0.1 to 1000 ng or advantageously 10-200 ng; and

IGFBP: 1 to 1000ng or advantageously 30-500 ng.

As will be described in more detail hereinafter, additional biologically active proteins such as EGF and bFGF may be present at 0.1 to 1000 ng or advantageously 10-200 ng.

Suitably, the culture medium and system of the invention comprise other defined components. Non-limiting and in some cases optional components include well known basal media such as DMEM or Ham's media, antibiotics such as streptomycin or penicillin, amino acid supplements such as L-glutamine, anti-

oxidants such as β-mercaptoethanol, buffers such as HEPES and a source of carbon dioxide as typically provided by cell culture incubators.

The invention also contemplates use of additional biologically active proteins that regulate cell growth, differentiation, survival and/or migration such as epidermal growth factor (EGF; Heldin *et al.*, 1981, Science **4** 1122-1123), fibroblast growth factor (FGF; Nurcombe *et al.*, 2000, J. Biol. Chem. **275** 30009-30018), basic fibroblast growth factor (bFGF; Taraboletti *et al.*, 1997, Cell Growth. Differ. **8** 471-479), osteopontin (Nam *et al.*, 2000, Endocrinol. **141** 1100), thrombospondin-1 (Nam *et al.*, 2000, *supra*), tenascin-C (Arai *et al.*, 1996, J. Biol. Chem. **271** 6099), PAI-1 (Nam *et al.*, 1997, Endocrinol. **138** 2972), plasminogen (Campbell *et al.*, 1998, Am. J. Physiol. **275** E321), fibrinogen (Campbell *et al.*, 1999, J. Biol. Chem **274** 30215), fibrin (Campbell *et al.*, 1999, *supra*) or transferrin (Weinzimer *et al.*, 2001, J. Clin. Endocrinol, Metab. **86** 1806).

Preferred additional biologically active proteins are EGF and bFGF.

In a particular embodiment, the invention contemplates use of any growth factor with a heparin-binding-like domain.

In another particular embodiment, the invention contemplates use of LIF and/or other agents that inhibit cell differentiation in addition to isolated protein complexes.

In yet another particular embodiment, the invention contemplates use of one or more of fibronectin, laminin, poly-L-lysine and poly-L-arginine in the culture medium, system and/or method of the invention.

The present invention also contemplates use of isolated protein complexes in the form of "*chimeric proteins*" comprising at least an integrin-receptor binding domain of VN and type I IGF receptor receptor-binding domain of IGF-I or IGF-II.

Preferably, the chimeric protein comprises a contiguous sequence of amino acids derived from an integrin-receptor binding domain of VN and a type 1 IGF receptor-binding domain of IGF-I or IGF-II.

In one particular embodiment, the chimeric protein contains full length mature VN and IGF-I proteins.

VN(1...459):IGF-I(1...70)

In another particular embodiment, the chimeric protein contains the mature VN protein with a deletion of residues 380 to 459 (C-terminal 80 amino acids), which is proposed to be more compact yet of equivalent function to a full-length VN:IGF-I fusion.

VN(1...379):IGF-I(1...70)

In yet another particular embodiment, the chimeric protein comprises the Somatomedin B domain of VN linked to IGF-I. This chimera would not interact with components in the ECM such as collagen and glycosaminoglycans. This incorporates a deletion of residues 53 to 459 on VN (connecting region, central beta-propeller domain and heparin binding domain)

VN(1...52):IGF-I(1...70)

In another particular embodiment, the chimeric protein of the invention comprises a Somatomedin B domain of VN as well as the connecting region created by deletion of residues 131 to 459 on VN.

VN(1...130):IGF-I(1...70)

In still yet another embodiment, a shorter chimeric protein retains the ligand binding regions within the Somatomedin B domain, polyanionic connecting region and polycationic heparin binding domain of VN that would, however, be unable to self associate. This involves a deletion of residues 131 to 346 on VN (central beta-propeller domain).

VN(1...130,347-459):IGF-I(1...70)

In a further embodiment, the chimeric protein comprises the most compact form of VN capable of binding its extracellular receptor. The protein has both the central domain and the C-terminal 80 amino acids of VN removed. This requires a deletion of residues 131 to 346 and 380 to 459 on VN (central beta-propeller domain and C-terminal 80 amino acids respectively)

VN(1...130,347-379):IGF-I(1...70)

In a still further embodiment, the chimeric protein comprises a C-terminal truncated VN without the heparin binding domain. Thus the protein contains the Somatomedin B domain, connecting region and central beta-propeller domain of VN and would therefore not interact with heparin and heparan sulfates. This chimera has a deletion of residues 347 to 459 on VN (heparin binding domain).

VN(1...346):IGF-I(1...70)

In alternative embodiments, the chimeric protein may comprise particular amino acid mutations or substitutions with respect to VN and growth factor amino acid sequences.

For example, it is proposed that chimeric proteins having one or more mutations that would either promote or inhibit the activation of the PI3-K pathway following binding to the $\alpha v\beta 3$ integrin. Thus a chimeric molecule with T50A and

T57A substitutions on VN would be analogous to a casein kinase 2 (CK2) non phosphorylated VN and be restricted to signalling through the ERK pathway (A) whereas synthetic constructs with the T50E and T57E substitutions on VN would mimic the CK2-phosphorlated VN and be capable of activating both the ERK and PI3-K pathway leading to altered intracellular signalling (B).

A) VN(T50A,T57A):IGF-I

B) VN(T50E,T57E):IGF-I

In other alternative embodiments are provided a chimeric protein containing both a S378E mutation on VN to inhibit PAI-1 binding by the chimera (C) and a S378A mutation on VN to promote PAI-1 binding and stabilisation within the chimeric protein (D). Furthermore, a S378A mutation may enhance cell migration.

C) VN(S378E):IGF-I

D) VN(S378A):IGF-I

In yet another alternative embodiment, the chimeric protein comprises a S362E substitution to mimic the phosphorylated serine and to consequently inhibit cleavage of the synthetic protein by plasmin.

VN (S362E):IGF-I

In a further alternative embodiment, the chimeric protein is a VN:IGF-I chimera comprising an N-terminal-truncated IGF-I to eliminate the possibility that IGFBPs could bind to IGF-I and consequently inhibit the biological activity of the VN:IGF-I chimeric protein. This construct includes a deletion of residues 1 to 3 on IGF-I (IGFBP binding region)

VN:IGF-I(4...70)

It will also be appreciated that according to other embodiments, the invention provides the fusion of VN with other peptide growth factors. Examples are provided by the following synthetic proteins.

VN:PDGFα(1...210) (NCBI accession # P04085)

VN:VEGF(1...102) (NCBI accession # 2VPFE)

It will also be appreciated that the chimeric proteins of the invention may further comprise a glycine/serine linker to bridge VN with IGF-I, VEGF or PDGF. Enzymatic linkers such as MMP-3 have recently been successfully introduced into chimeric proteins (eg. the use of an MMP cleavage site has been used to release functional VEGF from fibrin (J Control Release 2001 72:101-13)) and these may also be used to bridge VN with IGF-I, VEGF or PDGF.

The invention also extends to biologically-active fragments of the proteins of the invention. In one embodiment, said "*biologically-active fragment*" has no less than 1%, preferably no less than 10%, more preferably no less than 25% and even more preferably no less than 50% of a biological activity of a synthetic modulator protein of the invention.

Also contemplated are variant proteins and nucleic acids of the invention. In one embodiment, a "*variant*" synthetic protein of the invention has one or more amino acids that have been replaced by different amino acids. It is well understood in the art that some amino acids may be changed to others with broadly similar properties without changing the nature of the activity of the protein (conservative substitutions).

Variants may fall within the scope of the term "*homologs*" of synthetic proteins of the invention.

As generally used herein, a "*homolog*" shares a definable nucleotide or amino acid sequence relationship with a synthetic nucleic acid or protein of the invention as the case may be.

In one embodiment, a protein homolog of the invention shares at least 70%, preferably at least 80% and more preferably at least 90% sequence identity with the amino acid sequences described herein.

Preferably, sequence identify is measured over at least 60%, more preferably at least 75%, even more preferably at least 90% and advantageously over substantially the full length of the synthetic protein of the invention.

In order to determine percent sequence identity, optimal alignment of amino acid and/or nucleotide sequences may be conducted by computerised implementations of algorithms (Geneworks program by Intelligenetics; GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA, incorporated herein by reference) or by inspection and the best alignment (i.e., resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul et al., 1997, Nucl. Acids Res. 25 3389, which is incorporated herein by reference.

In another example, "sequence identity" may be understood to mean the "match percentage" calculated by the DNASIS computer program (Version 2.5 for

windows; available from Hitachi Software engineering Co., Ltd., South San Francisco, California, USA).

A detailed discussion of sequence analysis can be found in Unit 19.3 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel et al. (John Wiley & Sons Inc NY, 1995-1999).

The invention also contemplates derivatives of the synthetic protein of the invention. As used herein, "*derivative*" proteins of the invention have been altered, for example by addition, conjugation or complexing with other chemical moieties or by post-translational modification techniques as are well understood in the art

"*Additions*" of amino acids may include fusion of the polypeptides or variants thereof with other polypeptides or proteins. The other protein may, by way of example, assist in the purification of the protein. For instance, these include a polyhistidine tag, maltose binding protein, green fluorescent protein (GFP), Protein A or glutathione S-transferase (GST).

Other derivatives contemplated by the invention include, but are not limited to, modification to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the polypeptides, fragments and variants of the invention. Non-limiting examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by acylation with acetic anhydride; acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; amidination with methylacetimidate; carbamoylation of amino groups with cyanate; pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$; reductive

alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; and trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS).

Sulphydryl groups may be modified by methods such as performic acid oxidation to cysteic acid; formation of mercurial derivatives using 4-chloromercuriphenylsulphonic acid, 4-chloromercuribenzoate; 2-chloromercuri-4-nitrophenol, phenylmercury chloride, and other mercurials; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; carboxymethylation with iodoacetic acid or iodoacetamide; and carbamoylation with cyanate at alkaline pH.

The imidazole ring of a histidine residue may be modified by N-carbethoxylation with diethylpyrocarbonate or by alkylation with iodoacetic acid derivatives.

Examples of incorporating non-natural amino acids and derivatives during peptide synthesis include but are not limited to, use of 4-amino butyric acid, 6-aminohexanoic acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 4-amino-3-hydroxy-6-methylheptanoic acid, t-butylglycine, norleucine, norvaline, phenylglycine, ornithine, sarcosine, 2-thienyl alanine and/or D-isomers of amino acids.

Further examples of chemical derivatization of proteins are provided in Chapter 15 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et. al.*, John Wiley & Sons NY (1995-2001).

Isolated protein complexes inclusive of chimeric proteins of the invention (inclusive of fragments, variants, derivatives and homologs) may be prepared by any suitable procedure known to those of skill in the art.

In one embodiment, proteins of the invention are produced by chemical synthesis. Chemical synthesis techniques are well known in the art, although the skilled person may refer to Chapter 18 of CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et. al.*, John Wiley & Sons NY (1995-2001) for examples of suitable methodology.

In another embodiment, the protein may be prepared as a recombinant protein.

Production of recombinant proteins is well known in the art, the skilled person may refer to standard protocols as for example described in Sambrook *et al.*, MOLECULAR CLONING. A Laboratory Manual (Cold Spring Harbor Press, 1989), incorporated herein by reference, in particular Sections 16 and 17; CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel *et al.*, (John Wiley & Sons, Inc. 1995-1999), incorporated herein by reference, in particular Chapters 10 and 16; and CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, Inc. 1995-1999) which is incorporated by reference herein, in particular Chapters 1, 5 and 6.

Recombinant proteins of the invention may further comprise a fusion partner.

Well known examples of fusion partners include, but are not limited to, glutathione-S-transferase (GST), Fc portion of human IgG, maltose binding protein (MBP) and hexahistidine (HIS_6), which are particularly useful for isolation of the fusion protein by affinity chromatography. For the purposes of fusion protein

purification by affinity chromatography, relevant matrices for affinity chromatography are glutathione-, amylose-, and nickel- or cobalt-conjugated resins respectively. Many such matrices are available in "kit" form, such as the QIAexpress™ system (Qiagen) useful with (HIS_6) fusion partners and the Pharmacia GST purification system.

In some cases, the fusion partners also have protease cleavage sites, such as for Factor X_a or Thrombin, which allow the relevant protease to partially digest the fusion protein of the invention and thereby liberate the recombinant polypeptide of the invention therefrom. The liberated protein can then be isolated from the fusion partner by subsequent chromatographic separation.

Fusion partners according to the invention also include within their scope "*epitope tags*", which are usually short peptide sequences for which a specific antibody is available. Well known examples of epitope tags for which specific monoclonal antibodies are readily available include c-myc, haemagglutinin and FLAG tags.

Suitable host cells for expression may be prokaryotic or eukaryotic, such as *Escherichia coli* (DH5α for example), yeast cells, Sf9 cells utilized with a baculovirus expression system, CHO cells, COS, CV-1, NIH 3T3 and HEK293 cells, although without limitation thereto.

Keratinocytes engineered to express recombinant IGF, IGFBPs, VN or chimeric proteins

The invention further contemplates a keratinocyte or keratinocyte progenitor cell capable of expressing at least one recombinant protein selected from the group consisting of:

(i) a recombinant IGF;

(ii) a recombinant IGFBP;

(iii) a recombinant vitronectin;

(iv) a recombinant chimeric protein comprising at least a type-1 IGR-binding fragment of IGF-I or IGF-II; and

(v) a recombinant chimeric protein comprising at least an α-v integrin binding fragment of VN

Suitably, the recombinant proteins set forth in (i) to (iii) expressed by said keratinocyte are capable of forming an isolated protein complex comprising:

(a) IGF-I, an IGFBP and vitronectin; or

(b) IGF-II and vitronectin.

According to this aspect of the invention, paracrine/autocrine expression of IGFs, VN and/or IGFBPs may enable keratinocytes or keratinocyte progenitors to be cultured in media without serum or feeder cells and without the need to add isolated protein complexes to the culture medium. Alternatively, one or another component proteins of the isolated protein complex may be expressed by the keratinocyte or keratinocyte progenitor, other component proteins being provided in the culture medium.

Recombinant protein expression may be achieved by introduction of an expression construct into a keratinocyte or keratinocyte progenitor cell.

Typically, the expression construct comprises a nucleic acid to be expressed (encoding the recombinant protein) operably linked or operably connected to a promoter.

The promoter may be constitutive or inducible.

Constitutive or inducible promoters include, for example, tetracycline-repressible, ecdysone-inducible, alcohol-inducible and metallothionin-inducible promoters. Promoters may be either naturally occurring promoters (*e.g.* alpha crystallin promoter, ADH promoter, phosphoglycerate kinase (PGK), human elongation factor α promoter and viral promoters such as SV40, CMV, HTLV-derived promoters), or synthetic hybrid promoters that combine elements of more than one promoter (*e.g.* SR alpha promoter).

In a preferred embodiment, the expression vector comprises a selectable marker gene. Selectable markers are useful whether for the purposes of selection of transformed bacteria (such as *bla*, *kanR* and *tetR*) or transformed mammalian cells (such as hygromycin, G418 and puromycin).

Expression constructs may be introduced into keratinocyte or keratinocyte progenitor cells by well known means such as electroporation, microparticle bombardment, virus-mediated gene transfer, calcium phosphate precipitation, DEAE-Dextran, cationic liposomes, lipofectin, lipofectamine and the like, although without limitation thereto.

For non-limiting particular examples of methodology potentially applicable to expression of recombinant growth factor proteins in keratinocytes, reference may be made to Supp *et al.*, 2000, J. Invest. Dermatol. **114** 5 and Supp *et al.*, 2000, Wound Repair Regen. **8** 26-35.

Pharmaceutical compositions

The invention also provides pharmaceutical compositions that comprise an IGF protein complex of the invention and a plurality of keratinocytes in a

pharmaceutically acceptable carrier diluent or excipient, wherein the keratinocytes have been cultured using the culture medium, system or method of the invention.

Pharmaceutical compositions of the invention may be used to promote or otherwise facilitate cell migration, tissue regeneration and wound healing.

Generally, the compositions of the invention may be used in therapeutic or prophylactic treatments as required. For example, pharmaceutical compositions may be applied in the form of therapeutic or cosmetic preparations for skin repair, wound healing, healing of burns and other dermatological treatments.

Suitably, the pharmaceutical composition comprises an appropriate pharmaceutically-acceptable carrier, diluent or excipient.

Preferably, the pharmaceutically-acceptable carrier, diluent or excipient is suitable for administration to mammals, and more preferably, to humans.

By "*pharmaceutically-acceptable carrier, diluent or excipient*" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. Depending upon the particular route of administration, a variety of carriers, well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline and salts such as mineral acid salts including hydrochlorides, bromides and sulfates, organic acids such as acetates, propionates and malonates and pyrogen-free water.

A useful reference describing pharmaceutically acceptable carriers, diluents and excipients is Remington's Pharmaceutical Sciences (Mack Publishing Co. N.J. USA, 1991) which is incorporated herein by reference.

Any safe route of administration may be employed for providing a patient with the composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular, transdermal and the like may be employed.

Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, suppositories, aerosols, transdermal patches and the like. These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of the therapeutic agent may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

The above compositions may be administered in a manner compatible with the dosage formulation, and in such amount as is pharmacuetically-effective. The dose administered to a patient, in the context of the present invention, should be sufficient to effect a beneficial response in a patient over an appropriate period of time. The quantity of agent(s) to be administered may depend on the subject to be treated inclusive of the age, sex, weight and general health condition thereof, factors that will depend on the judgement of the practitioner.

With regard to pharmaceutical compositions for wound healing, particular reference is made to U.S. patent 5,936,064 and International Publication WO99/62536 which are incorporated herein by reference.

Preferably, the composition of the invention is suitable for spray delivery *in situ*.

The term "*spray*" encompasses and includes terms such as "*aerosol*" or "*mist*" or "*condensate*" that generally describe liquid suspensions in the form of droplets.

Although not wishing to be bound by any particular theory, the invention contemplates that the inherent "stickiness" of VN in IGF complexes present in the spray composition will facilitate delivery of growth factors such as IGF-I, IGF-II and other growth factors such as EGF and FGF.

Typically, spray compositions of the invention will be delivered by apparatus such as a pressurised canister equipped with a delivery outlet.

An example of an aerosolised keratinocyte delivery systems such as for wound healing in a pig model, is provided by Navarro *et al.*, 2000, J Burn Care Rehabil **21** 513. Reference is also made to Grant *et al.*, 2002, Br J Plast Surg **55** 219 which describes use of aerosolised keratinocytes in conjunction with fibrin glue for wound healing in a pig model.

Therapeutic uses

The invention provides a culture medium, system and method for propagating primary keratinocytes *ex vivo*.

Furthermore, the present invention provides methods of treating burns, wounds and ulcers as well as methods that relate to cosmetic skin treatments to improve or enhance skin quality or skin appearance.

These methods are particularly aimed at treatment of mammals, and more particularly, humans.

Such methods include administration of pharmaceutical compositions as hereinbefore defined, and may be by way of microneedle injection into specific tissue sites, such as described in U.S. patent 6,090,790, topical creams, lotions or sealant dressings applied to wounds, burns or ulcers, such as described in U.S. patent 6,054,122 or implants which release the composition such as described in International Publication WO99/47070.

There also exist methods by which skin cells can be genetically modified for the purpose of creating skin substitutes, such as by genetically engineering desired growth factor expression (Supp *et al.*, 2000, J. Invest. Dermatol. **114** 5). An example of a review of this field is provided in Bevan *et al.*, Biotechnol. Gent. Eng. Rev. **16** 231.

Also contemplated is "seeding" a recipient with transfected or transformed cells, such as described in International Publication WO99/11789.

These methods can be used to stimulate cell migration and thereby facilitate or progress wound and burn healing, repair of skin lesions such as ulcers, tissue replacement and grafting such as by *in vitro* culturing of autologous skin, re-epithelialization of internal organs such as kidney and lung and repair of damaged nerve tissue.

Skin replacement therapy has become well known in the art, and may employ use of co-cultured epithelial/keratinocyte cell lines, for example as described in Kehe *et al.*, 1999, Arch. Dermatol. Res. **291** 600 or *in vitro* culture of primary (usually

autologous) epidermal, dermal and/or keratinocyte cells. These techniques may also utilize engineered biomaterials and synthetic polymer "scaffolds".

Examples of reviews of the field in general are provided in Terskikh & Vasiliev, 1999, Int. Rev. Cytol. **188** 41 and Eaglestein & Falanga, 1998, Cutis **62** 1.

More particularly, the production of replacement oral mucosa useful in craniofacial surgery is described in Izumi *et al.*, 2000, J. Dent. Res. **79** 798. Fetal keratinocytes and dermal fibroblasts can be expanded *in vitro* to produce skin for grafting to treat skin lesions, such as described in Fauza *et al.*, J. Pediatr. Surg. **33** 357, while skin substitutes from dermal and epidermal skin elements cultured *in vitro* on hyaluronic acid-derived biomaterials have been shown to be potentially useful in the treatment of burns (Zacchi *et al.*, 1998, J. Biomed. Mater. Res. **40** 187).

Polymer scaffolds are also contemplated for the purpose of facilitating replacement skin engineering, as for example described in Sheridan *et al.*, 2000, J. Control Release **14** 91 and Fauza *et al.*, 1998, *supra*, as are microspheres as agents for the delivery of skin cells to wounds and burns (LaFrance & Armstrong, 1999, Tissue Eng. **5** 153).

Keratinocyte sheets typically produced for therapeutic use are responsible for the ultimate closure of burn wounds. This sheet graft technique is applicable to all partial thickness burn injuries and is most useful in treating large surface area wounds where early permanent closure of both wound and donor sites is nearly impossible without external help. This is the type of injury responsible for the death of patients burnt in the recent Bali bombing.

Currently, it is possible to grow enough skin from a patient skin biopsy the size of a fifty-cent piece to cover an entire adult. This culture process takes 17 days.

However, earlier skin replacement is urgently needed to reduce patient trauma, risk of infection, scarring and the present requirement for expensive temporary skin replacements ahead of permanent skin grafting. In addition, a sheet of cultured skin comprises many skin cells, some mature and some immature. The simple act of allowing cultured keratinocytes to reach confluence (necessary to produce sheets of skin) causes cells to prematurely loose their primitive characteristics *i.e* to differentiate. When a sheet of cultured skin is applied, only the immature cells are capable of attaching and establishing themselves on the patient. Because only small areas adhere, the sheets are very susceptible to damage arising from friction or movement of the patient and can sometimes result in the loss of the entire graft. Furthermore, in a sheet graft, the more mature skin cells in the sheet, the more likely it will be that the graft will not take and the cells themselves will not proliferate and migrate on the wound bed itself. Thus it is clear that earlier application of immature skin cells will result in better graft take and reduce scarring.

The present invention therefore provides a spray or aerosol delivery method to deliver skin cells cultured *ex vivo* onto a patient's burnt, ulcerated or wounded skin to enable a larger surface area of the patient's body to be covered by immature skin cells much earlier than existing sheet graft technology. This could be as early as only 7 days. This would also significantly reduce scar formation, shock and heat loss and would enable faster return of skin function in partial thickness and also full thickness burns.

According to the invention, the spray or aerosol may further comprise isolated protein complexes comprising IGFs, VN and IGFBPs together EGF and/or bFGF to promote skin cell growth and migration *in situ*.

The patients' own skin cells (autologous skin) and donor skin cells (allogeneic skin) can be grown and used for early burn closure. Donor cells do not express transplantation antigens, so they do not cause an immune response in the patient. The donor skins cells, however, are eventually replaced by the patients' own skin cells.

Although autologous cells are preferred, use of allogeneic cells in a spray-on-skin would allow immediate application to a needy patient. Alternatively, sufficient autologous skin cells could be cultured in approximately seven days for use in a therapeutic spray.

Another treatment contemplated by the present invention is the treatment of burns patients to achieve early closure of full thickness wounds, because take of cultured skin on a wound that has removed both the surface (epidermal) and deep layer (dermis) of skin is poor. The invention contemplates use of dermal substitutes in conjunction with the spray-on-skin to effect early permanent closure of these most horrific injuries. Both biological and synthetic dermal substitutes are contemplated. For example, the a de-epidermised, de-cellularised cadaveric-derived dermal scaffold comprising isolated protein complexes of the invention overlayed with a synthetic epidermis (dressing). After approximately 7 days the dermis we hypothesise that this dermis will be highly infilitrated by autologous endothelial cells. At this time, the synthetic dermis will be removed and the patient's own ex-vivo expanded fibroblasts and keratinocytes will be applied to the allo-dermis.

It is anticipated that the spray-on-skin, rather than epidermal sheets, will be successful as the dermal substitute will act as a nutritious stabilising scaffold promoting the migration and anchoring of skin cells and other important cells

normally found in the skin. This will result in improved take of cultured skin cells in full thickness skin injuries

So that the present invention may be more readily understood and put into practical effect, the skilled person is referred to the following non-limiting examples.

EXAMPLES

EXAMPLE 1

MATERIALS AND METHODS

PRIMARY KERATINOCYTE CULTURE

Keratinocytes are isolated from adult human skin using the standard procedures essentially the same as that originally reported by Rheinwald & Green, 1977, Nature **265** 421. Briefly this involves digestion of the skin sample for one hour at 37° C in Dispase II solution. The recovered epithelium is subsequently digested for a further 10 minutes at 37° C with 0.25% trypsin/0.02% EDTA to dissociate the cells. Residual trypsin activity is inactivated and recovered cells are then washed and co-seeded into tissue culture dishes containing lethally irradiated 3T3 mouse fibroblasts. "Control" cells, cultivated using these standard conditions are grown in DMEM/F12 medium supplemented with 10% fetal calf serum, 0.1% penicillin-streptomycin solution, 0.4 µg/ml hydrocortisone, 0.1 µg/ml cholera toxin, 10 ng/ml human recombinant epidermal growth factor (EGF), 5 µg/ml insulin, 5 µg/ml transferrin and 2 nM tri-iodothyronine, while cells treated with isolated growth factor complexes use identical media except that no insulin is present. Insulin is not included in media used in conjunction with isolated protein complex treatments to minimize competitive binding of insulin to the type-1 IGF

receptor. Cells cultured on isolated growth factor complex-coated dishes also differ from those cultured following the standard procedure in that the cells will be seeded onto plates without irradiated mouse fibroblasts.

PREPARATION OF CULTURES

Three hundred microlitres of DMEM or DMEM/F12 media containing either with 10% fetal bovine serum or without serum was added to 24-well tissue culture dishes or to the lower chamber of a Transwell™ and incubated at 37° C for 2 hrs. Media containing unbound VN was removed and the wells were washed with 1 mL Hepes Binding Buffer (HBB). Three hundred µL HBB containing IGF-II or IGF-I + IGFBP (GroPep, Adelaide, SA, Australia) was then added and the plates incubated again for 2 hrs. The solution containing unbound IGFs and IGFBPs (if applicable) was removed and the wells were washed with HBB and air dried in laminar flow hoods.

PROTEIN SYNTHESIS ASSAY

Passage 3 human skin keratinocytes were derived from an adult skin biopsy and assessed for the stimulation of protein synthesis in the presence and absence of IGF + VN complexes. Here, 24 well plates were coated for 2 hours with 300 ng of vitronectin and then washed to remove unbound vitronectin. Wells were then incubated with the growth factors to be examined, that is; epidermal growth factor, basic fibroblast growth factor, insulin-like growth factor-I and insulin-like growth factor-II, in combination with insulin-like growth factor binding protein-5, were added to the wells and allowed to bind the vitronectin overnight. The next day the wells were washed twice to remove any unbound growth factors and the plates allowed to air dry. Keratinocytes were then harvested and seeded at a density of 1 x

10^5 cells/well in serum-free Dulbecco's Modified Eagle Medium (DMEM) along with 1 uCi/well of [^{3}H]-leucine. In select wells, cells were seeded in Defined Keratinocyte Medium (DKM) (Invitrogen), a commercially available product for the serum-free culture of keratinocytes. Plates were then incubated for 48 hours and then washed to remove any unincorporated [^{3}H]-leucine. Incorporated of [^{3}H]-leucine into *de novo* synthesised protein was determined by sampling solubilised protein precipitate for beta-scintillation counting.

MTT-ESTA ASSAY

Human keratinocytes were isolated and the cultures established using standard culture techniques of fully supplemented Greens Media with a feeder layer of lethally irradiated mouse 3T3 cells. Cells were expanded to passage 4 and seeded into 24 well plates in Greens media in the presence or absence of Fetal Calf Serum (FCS) and 3T3 cells. In select treatments, wells were coated with isolated protein complexes. Wells were incubated with 300 ng of vitronectin for 2 hours and then aspirated prior to the addition of insulin-like growth factor-I and either insulin-like growth factor-3 (FIG. 1A) or insulin-like growth factor-5 (FIG. 1B). Plates were incubated overnight and aspirated prior to seeding cells. The cultures were assessed for metabolic activity as measured using the MTT-esta assay as described previously (Ealey *et al.* 1988, J Mol Endocrinol 1:R1-R4.).

RESULTS

In view of the significant enhanced functional responses obtained with isolated growth factor complexes in cell lines (International Publication WO 02/24219; Noble *et al.*, 2003, *supra*; Kricker *et al.*, 2003, *supra*) we recently extended our studies to cultures of keratinocytes derived from adult skin. In particular

we examined the potential of isolated growth factor complexes to replace serum and feeder cells used in current best clinical practice for *ex vivo* expansion of keratinocytes for split thickness autografting. While this procedure has significantly advanced therapies available to burns patients, the culture of keratinocytes derived from patients is conducted in the presence of fetal bovine serum (FBS), a semi-defined xenobiotic product that is a potential source of pathogens. In addition, in the early stages of keratinocyte derivation and establishment a feeder layer of cells derived from a second species, namely murine 3T3 fibroblasts, is used as a source of cytokines and matrix elements to encourage cell attachment and growth. FBS also contributes to these effects.

As (i) IGFs account for a large proportion of the cytokines secreted by the feeder cells; (ii) we have established that VN replaces any requirement for serum to facilitate the attachment of primary cultured keratinocytes seeded at low density to plasticware; and iii) the effects we have obtained with keratinocyte cell lines cultured on isolated growth factor complexes are equivalent to those obtained with media containing 10% FBS (1), we hypothesised that isolated growth factor complex-supplemented media had the potential to provide a superior product for autologous keratinocyte engineering applications. This hypothesis is supported by the fact that IGFs are key mitogens that stimulate keratinocyte proliferation, yet keratinocytes themselves do not secrete IGF-I. While serum-free media, such as KGM™ (Clonetics) and EpiLife™ (Sigma-Aldrich), have been developed commercially for keratinocyte expansion, these media require the addition of bovine pituitary extract, which is also undefined, a xenobiotic and a potential source of pathogens, or alternatively, the addition of expensive supplements. Furthermore, most current

serum-free keratinocyte culture applications demand very high seeding densities which defeats the purpose of attempting to culture large quantities of keratinocytes rapidly and accounts for the poor adoption of these practices for routine clinical applications.

We have directly tested our hypothesis and the results are illustrated in Figure 1. In this experiment keratinocytes were derived from adult skin and established using usual procedures for 7 days. The cells were then passaged by trypsinisation and seeded at low density (8,500 keratinocytes/cm^2) on isolated growth factor complex-coated tissue culture plastic and grown in the absence of feeder cells, and minus both FBS and insulin (Fig. 1) for a further 7 days. Cells grown in these conditions were found to expand more rapidly than those grown using only current best clinical practice (*i.e.* grown in the presence of FBS and 3T3 mouse feeder fibroblasts; Fig. 1). The margins of the colony grown in the presence of isolated growth factor demonstrate keratinocytes that are outwardly mobile, healthy and proliferating. The innermost cells depicted in Fig. 1 show the typical pavement morphology observed in keratinocyte cultures near confluence, with confluence in this case obtained in just 7 days.

The effect of additional growth factors EGF amd bFGF is demonstrated in Figure 2. We examined passage 3 human skin keratinocytes (derived from an adult skin biopsy) and assessed the stimulation of protein synthesis by supplemented IGF + VN complexes over 48 hr. These treatments were tested in parallel with cells grown in Defined Keratinoctye-SFM (DKM) (Invitrogen), a commercially available product for the serum free culture of keratinocytes, containing undefined amounts of insulin, EGF and bFGF. DKM was found to stimulate increases in protein synthesis of 148%

above control wells (-VN), which was significantly higher ($p<0.05$) than the effect of VN alone (+VN) or the absence of VN and growth factors (-VN). The dimeric IGF-II + VN and trimeric IGF-II + VN + IGFBP-5 complexes also stimulated significant increases in protein synthesis of 134% and 161% respectively ($p<0.05$). Indeed there were no significant differences ($p>0.05$) in the stimulation of protein synthesis observed for DKM, dimeric and trimeric complexes, indicating that both complexes are equally efficient at stimulating keratinocyte protein synthesis as the commercially available DKM.

When EGF, bFGF, or both growth factors in combination, were added to the trimeric complex increases of 216%, 248% and 213% were observed. All of these responses were significantly higher than that of DKM ($p<0.05$). Likewise, when EGF, or both EGF and bFGF, were added to dimeric complexes, significant increases in protein synthesis of 192% and 198% respectively, were obtained which were also significantly higher than that of DKM ($p<0.05$). These results highlight that incorporating EGF and bFGF into isolated protein complexes stimulate increases in protein synthesis above that of a commercially available product for the serum-free and feeder-free cultivation of keratinocytes.

EXAMPLE 2

GROWTH AND MIGRATION OF ORS-DERIVED CELLS

Primary outer root sheath (ORS) cultures will be derived from anagen-phase hair follicles harvested from the scalp of consenting diabetic patients and cultured using the methods described by Limat & Hunziker, 2002, Cells Tissues Organs 172 79-85 and International Publication WO 01/59442. The cells will be *ex vivo* expanded using a pre-formed feeder layer of post-mitotic human dermal fibroblasts

and fetal calf serum supplemented media as described above for keratinocytes derived from skin. The cultures will be maintained in a sub-confluent state for a maximum of three passages and morphological and functional assessment of the growth of the cells in the presence of isolated protein complexes examined in the absence of serum and feeder cells.

The particular complexes determined to be optimal for skin-derived keratinocyte growth will be tested.

Having established that *ex vivo* expanded ORS-derived keratinocyte progenitor cells grow and migrate in the presence of isolated protein complexes, it will then be determined whether the initial derivation of the cells from the anagen-phase ORS and the subsequent primary culture can also be performed in serum- and feeder cell-free conditions. Thus the ORS of anagen-phase follicles will be explanted onto the microporous membranes of cell culture inserts and rather than coating the underside of the membrane inserts with a feeder layer of postmitotic dermal fibroblasts, the undersurface will instead be coated with isolated protein complexes. The cells will be grown in serum-free media alone, or media supplemented with autologous serum obtained from the patient, or media containing isolated protein complexes. The growth rate of the ORS-derived cells grown the presence of isoolated protein complexes will be compared with cells grown on inserts using standard procedures.

Epidermal equivalents will also be prepared by exposing the cells to air, as described by Limat & Hunziker, 2002, *supra*, and characterized using histological, ultrastructural (e.g. basement membrane-like structure, keratohyalin granules, keratinosomes) and immunohisto-chemical (e.g. keratins, integrins, gp80, involucrin,

filaggrin) criteria. If successful, use of ORS-derived progenitor cells with this growth factor + VN technology will not only significantly reduce manufacturing costs, but will also enhance safety, thus expedite regulatory issues associated with the approval of a cell-based therapeutic.

EXAMPLE 3

PREPARATION OF PURIFIED VITRONECTIN

Autologous VN purified patient blood (typically present at 0.4 mg/ml). will be used to support the growth of the patient's own keratinocytes *ex vivo*. We will evaluate monoclonal antibodies they have produced against vitronectin and that have successfully been used for purification of vitronectin from human serum (Underwood *et al.*, 2001, J Immunol Methods. **247** 217-24). The monoclonal antibodies selected for evaluation will be coupled to the support purification matrix using methodologies similar to those described by to purify VN from serum. At this stage we estimate we will need 0.25 mg of VN to culture 1 m^2 of patient cells and this should be readily obtained from 20 ml of patient blood. The purification procedure by Underwood *et al.*, 2001, *supra* will be modified with the emphasis being on minimal manipulation and simplicity: the aim being to develop a disposable affinity purification matrix that requires ideally, only 2-3 washing steps. As the VN will be from the patients themselves, the requirement for pure VN is reduced, provided that the VN obtained is able to still promote cell growth efficiently. Thus VN purified using the protocols developed will be evaluated for efficacy in promoting keratinocyte growth as well as through standard biochemical analyses such as SDS-PAGE, N-terminal protein sequencing, electrospray mass analysis, IGF- and IGFBP-binding, and will be compared with VN purchased from Promega Pty. Ltd.

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. It will therefore be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention.

All computer programs, algorithms, patent and scientific literature referred to herein is incorporated herein by reference.

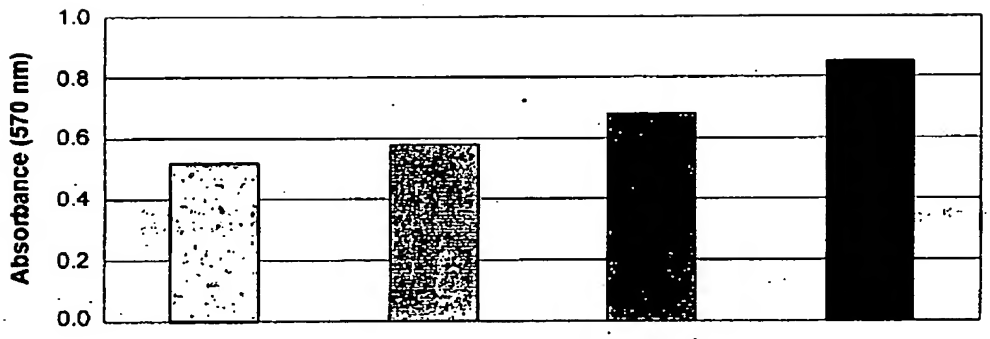
DATED this twenty-eighth day of July 2003

QUEENSLAND UNIVERSITY OF TECHNOLOGY

by its Patent Attorneys

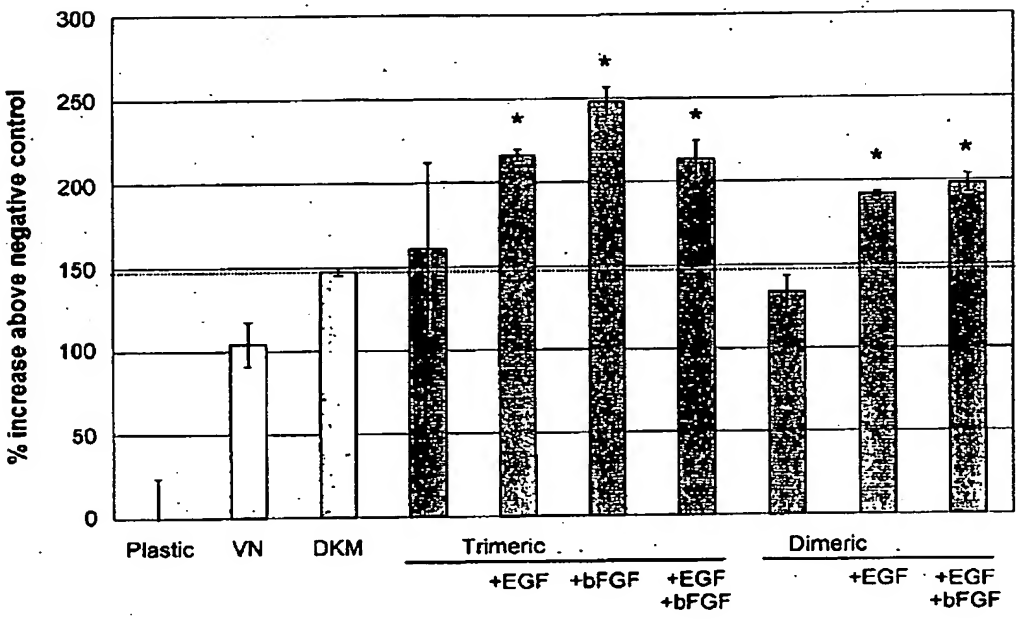
FISHER ADAMS KELLY

a)



b)

FIG. 1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200
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100
50
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Plastic
VN
DKM
Trimeric
+EGF
+bFGF
+EGF +bFGF
Dimeric
+EGF
+EGF +bFGF
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FIG. 2